DATA EVALUATION RECORD
FORAGING ACTIVITY – HONEYBEES
Apis mellifera
(NON-GUIDELINE STUDY)

1. **CHEMICAL:** Clothianidin PC Code No.: 044309

2. **TEST MATERIAL:** Poncho or Poncho Pro (FS 600) Purity: Not reported

3. **CITATION**

Authors:	Lückmann, J., S. Hofmann, M. Münderle and C. Garrido
Title:	Field Survey on Guttation of Maize Seedlings under Agronomic Use Conditions in Austria and Assessment of the Relevance of Guttation Fluid for Honeybees
Study Completion Date:	January 14, 2010
Laboratory:	RIFCon GmbH Im Neuenheimer Feld 517 69120 Heidelberg, Germany
Sponsor:	Bayer CropScience AG BCS-D-EnSa-ETX Alfred-Nobel-Str. 50 40789 Monheim, Germany
Laboratory Report ID:	R09105
MRID No.:	479753-01
DP Barcode:	D374484

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Signature: **Date:** 7/12/11

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Signature: **Date:** 8/4/11

5. **APPROVED BY:** {……}, {Specialty}, OPP/EFED/ERB-{Section}

Signature: **Date:**

6. STUDY PARAMETERS

Test Species: Honeybees (*Apis mellifera* L.; Hymenoptera, Apoidea). Healthy colonies were used, and consisted of one egg-laying queen and approximately 10,000-20,000 bees per colony.
Age of Test Organism at Test Initiation: Not specified. Hive colonies included one egg-laying queen, and nominally 10,000-20,000 bees of mixed sex and age.
Test Duration: Six weeks.

7. CONCLUSIONS:

In a 3 or 6 week field test, honeybees were exposed to guttation fluid from clothianidin-treated maize seeds under commercial conditions using the formulations Poncho or Poncho Pro (FS 600) at a nominal application rate of 0.5 or 1.25 g clothianidin/seed, respectively, in two regions in Austria (Baumgartenberg and Jennersdorf). Each region contained 15 test fields with two test colonies per field. The objective was to evaluate the association of guttation with honeybee flight activity and to monitor the use of guttation fluid by honeybees. Drinking troughs were placed next to colonies at 5 out of 15 fields in each region. Observations were made for guttation fluid and/or dew, bee behavior and foraging activity, and mortality of the bees twice daily. Colony strength and development was also assessed immediately after colony set-up and 3 weeks later by evaluating bee health, number of combs with bees and brood and the size and condition of the brood nest. At the end of exposure the colonies were relocated to an unexposed bee yard and the final check after 3 additional weeks. During the exposure phase, guttation fluid and dead bee samples were collected for residue analysis of the test material and its metabolites.

Guttation coincided with honeybee activity in the morning averaging 85%. The overlap between guttation and honeybee activity was lower in the evening, averaging 37%. Therefore, the coincidence of guttation of maize seedlings and honeybee activity in the morning was common and lasted up to 6 weeks after seedling emergence, while the coincidence was less regular in the evening with honeybees looking for water sources in the nearest vicinity of the hives.

There were no differences in colony growth associated with access to drinking troughs during the exposure period ($p>0.05$; Mann-Whitney U-test). However, significant differences were noted between the two test regions. In Jennersdorf, the effects of starvation on colonies due to unfavorable apicultural landscape conditions were clear. Even after a 3 week recover period honeybee colonies from Jennersdorf did not reach the level of those from Baumgartenberg at the end of exposure, emphasizing the importance of appropriate nutritional supply.

A period of higher bee mortality was noted *ca*. 30 days after drilling; however, colony development was not affected. The number of days with greater mortality is more frequent for hives with no access to an additional water source ($Chi^2 = 5.8$, $p<0.05$). However, this correlation was not found for both regions. In Baumgartenberg, the difference between colonies

with and without drinking troughs was highly significant ($Chi^2 = 10$, $p<0.001$), but there was no significant difference observed for Jennersdorf ($Chi^2 = 0.02$, $p>0.05$). Residues were found in colonies with and without water trays; however, residues were not correlated with the number of dead bees in the traps.

Some of the guttation fluid samples were assumed to be a mix of guttation fluid, dew and/or rain. Residue analysis indicated initial concentrations for both regions were between 100 to 200 mg/L clothianidin. Residue levels in all samples declined exponentially over time with concentrations in guttation fluid of about 1 mg/L three weeks after emergence and 0.1 mg/L within five weeks

In Baumgartenberg, clothianidin residues in bees were between <LOD and 45.5 ppb and in guttation water were between <LOQ and 717 mg/L. TZNG residues in bees were between <LOD and 31.2 ppb and in guttation water were between <LOD and 4.0 mg/L. TZMU residues in bees were between<LOD and 3.3 ppb and in guttation water were between <LOD and 9.0 mg/L. TMO residue levels in bees and guttation water were always below LOQ.

In Jennersdorf, clothianidin residues in bees were between <LOD and 384.9 ppb and in guttation water were between <LOQ and 285 mg/L. TZNG residues in bees were between <LOD and 39.7 ppb and in guttation water were between <LOD and 4.9 mg/L. TZMU residues in bees were between<LOD and 12.4 ppb and in guttation water were between <LOD and 6.7 mg/L. TMO residue levels in bees were always below LOQ and in guttation water were between <LOD and 0.054 mg/L.

The study author concluded that guttation in maize seedlings occurred regularly and the presence of guattation fluid usually overlapped with bee flight activity. Initial residues of clothianidin in guttation fluid from treated seeds were in the range of 100 to 200 mg/L, but decreased exponentially in the following weeks, reaching levels of 1 mg/kg after 3 weeks and were below 0.1 mg/L after 5 weeks. Bee colonies were very adaptable and developed reasonably well despite low population strength at the start of the experiment. Even transitory starvation stress in Jennersdorf did not ultimately affect the majority of managed colonies. However, low population strength in the early phase, suboptimal forage conditions, and some infestation of diseases made these colonies more susceptible to chemical stress factors than typical commercial colonies in more appropriate apicultural conditions. Nevertheless, colony development was not affected by exposure to guttation in Poncho- or Poncho Pro-treated maize seedlings. After removing the colonies from experimental conditions, development continued as expected with colonies from Jennersdorf region starting to recover from food scarcity. Therefore, the study authors concluded that no visible harmful effect on the colony level due to exposure to guttating maize fields sown with clothianidin-treated seeds. The honeybees were able to develop properly even under unfavorable conditions of the study.

The reviewer agrees with the study author that exposure of honeybees to guttation fluid from clothianidin-treated maize seeds had no harmful effects at the colony level over the test period of

3 or 6 weeks. However, the test material did have detrimental effects on individuals with one case of increased aggression in two colonies from one field and some evidence of colony weakening due to increased infestation and disease. Furthermore, the study period may not have been adequate to accurately determine colony effects over time under field conditions in which exposure may have occurred for a longer period of time. Additionally, recovery under conditions of a non-exposure bee yard are not available

8. ADEQUACY OF THE STUDY

A. Classification: Core/Supplemental/Invalid

B. Rationale:

C. Repairability:

9. GUIDELINE DEVIATIONS: This is a non-guideline test.

10. SUBMISSION PURPOSE: This study was submitted to survey guttation behavior of maize seedlings (variety not reported) which were grown from clothianidin-treated seeds under commercial conditions using the formulations Poncho or Poncho Pro (FS 600) at a nominal application rate of 0.5 or 1.25 g clothianidin/seed, respectively. The objective was to evaluate the association of guttation with honeybee flight activity and to monitor the use of guttation fluid by honeybees.

11. MATERIALS AND METHODS

Test Material

The test material Poncho or Poncho Pro was a flowable concentrate for seed treatment (FS 600; formulation concentrations not reported) applied at concentration of 0.5 or 1.25 g clothianidin/seed, respectively, which corresponded to 50 or 125 g a.s./ha, respectively (also 83.3 g product/ha or 208.3 g product/ha). Based on the nominal dose rate and the typical local drilling rates for the Baumgartenberg and Jennersdorf test regions, the application rates were between 39.5-44.0 g clothianidin/ha and 40.0-100.0 g clothianidin/ha, respectively. Solubility of clothianidin is 327 mg/L (at 20°C) in pure water. Description and storage of the test material was not provided.

Test Organisms

Healthy honey bee (*Apis mellifera*) colonies purchased from a professional bee keeper were located directly adjacent to (no more than 30 cm from the edge) or on each maize test fields at least 3 days after sowing in most cases. Two colonies were located in each field and monitored for brood and colony development. A description of the colonies at study initiation was not reported. The study was carried out during the seasonal growth phase (period of maximum brood activity) and therefore a period of high water demand. All bee colonies development was estimated according to the Liebefeld method. The number of bees is assessed by the area of the comb covered by bees. The experimental colonies had Zander combs, corresponding to eight comb areas (100 cm^2 each) on each side and every fully covered comb area corresponds to 130 adult bees. The number of brood cells is estimated using the same method, each area corresponding to 400 cells in the case of worker brood and 230 cells in the case of drone brood. The first estimation was recorded at the setup of the colonies, and additional estimations every three weeks. Between the first two estimations, colonies were controlled weekly to evaluate bee health, brood status and the presence of the queens. Colony strength was also assessed by combs covered by bees, size of the brood nest, and brood stages noting the patchiness of the brood nest, symptoms of diseases and abnormal bee behavior. The area covered by brood was assessed by marks from 1 (small brood area) to 4 (brood covered whole comb) in the center of the brood nest. The brood stages were codified with 1 = eggs, 2 = larvae, and 3 = capped brood. These qualitative assessments allow recognition of any disease symptoms, swarming behavior or significant negative environmental effects.

At delivery, the average colony strength was 1,370 and 2,030 bees in Baumgartenberg and Jennersdorf, respectively. Due to these low starting populations, and to accomplish the objectives of the study, all colonies with population strengths of less than 1,000 bees were replaced. Since inoperative colony strength was discovered during the first assessment, before emergence, the uptake of guttation fluid was not a causative factor for the low colony strength.

Seed Treatment and Crop Maintenance

Maize seedlings were commercially dressed with Poncho or Poncho Pro, formulation type FS 600 containing clothianidin (purity not reported). The seeding rate was *ca.* 2 units/ha (1 unit = 50,000 maize seeds). The nominal seeding loading was 0.50 mg a.s./seed and 1.25 mg a.s./seed for Poncho and Poncho Pro, respectively; the nominal a.i. application rate was 50 g a.s./ha (corresponding to 83.3 g product/ha) and 125 g a.s./ha (corresponding to 208.3 g product/ha), respectively. No further treatment or crop maintenance details provided.

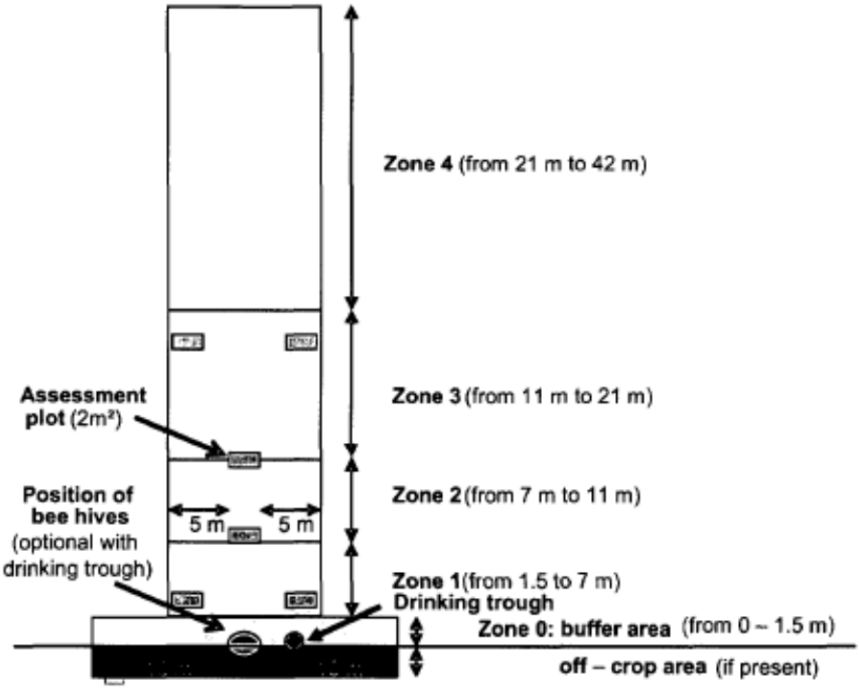
Test Design

The study was conducted in 15 commercially managed maize fields in each of two representative regions (each with 4-5 sub-regions) for commercial maize production in

Austria. One region was north of the Alps known as Baumgartenberg, and one region south of the Alps known as Jennersdorf. The study was designed to represent a worst case exposure and the sites were selected on the basis of representing typical maize cultivation area and nearly exclusive commercial sowing of neonicotinoid treated maize seeds; meteorological and pedological conditions that favored exudation of guttation fluid by maize seedlings; distant from permanent water source (≥300 m); and landscape that provided only limited nectar and pollen sources to enhance water demand of exposed bee colonies. The Baumgartenberg region was more richly structured with small agricultural croplands, and woods with edges of flowering shrubs and plants compared to the Jennersdorf region. In the Baumgartenberg region, honeybee forage conditions were acceptable but wild plant blooming was limited and it is not likely that the nectar flow of forage weeds significantly reduced the water need of the colonies in early morning. In the Jennersdorf region, forage conditions were poor for maintaining beehives, therefore to reduce starvation stress, all colonies were fed on May 9th with 1.25 kg of Apifonda, a pasty food intended to not reduce water demands of colonies and in fact enhanced the water needs of the colonies beyond the normal levels.

Two bee colonies were placed directly adjacent to or on each of the test fields with the front of the hive oriented parallel to the seedling rows if possible at least three days after sowing in most cases. The area in front of the hives was considered the most attractive to honeybees collecting water. Therefore, this area was monitored for the presence of guttation fluid and/or dew, the presence of honeybees sitting on the ground or on maize seedlings, the uptake of guttation fluid or dew, and bees showing unusual behavior. The assessment was divided in to five zones: zone 0 covered the surrounding area in front and next to the bee hives (0-1.5 m x 10 m); zones 1-4 covered the in-field assessment area further away from the hives and were all 5 m wide. Depths of zones into the test fields were: Zone 1 was 1.5-7 m, zone 2 was 7-11 m, zone 3 was 11-21 m and zone 4 was 21-42 m into the field. If present, an off-crop area (areas with grasses or herbal plants) along the edge of the field was assessed; additionally there were six 2 m^2 assessment sub-plots in zone 1-3.

Honeybee colony assessment scheme



To differentiate the effects of guttation fluids in the presence or absence of alternative water sources, 10 out of the 30 study fields (5 in each region) received water trays (with *ca*. 5 g common table salt/L) to simulate an alternative water source directly next to the hives. At each beehive, a dead bee trap was installed and emptied each day in the morning using featherweight forceps to avoid contamination. If more than 10 dead bees were sampled, they were analyzed for residue. If significant guttation was observed in the morning, up to 3 samples (*ca*. 1 mL) were collected with glass Pasteur pipettes and stored in Eppendorf vials (2 mL). Bees and guttation fluid samples were stored in a cooler and transferred within 10 hours to a deep freezer (-20°C). Samples were collected within the first hour of field inspection outside the assessment area and a distance of at least 20 m from the bee hives. For water in funnels formed by adjacent maize leaves, up to 3 separate water samples were collected and analyzed for clothianidin residues.

Assessment and monitoring activities

Monitoring began at emergence and continued for three successive weeks, with monitoring continuing at Baumgartenberg for another three weeks due to continuing guttation; prolonged monitoring in the Jennersdorf region was not possible due to unfavorable apidological conditions. Assessment began in early morning near sunrise with guttation checks in the field and neighboring fields or adjacent vegetation, dead bee traps were emptied, and precipitation was recorded. In the Baumgartenberg region, the honeybees were most active in the nearest vicinity of the colonies, therefore the

assessments were exclusively focused on the off-crop area, zone 0 and zone 1. Similar observations were made in the evening.

The proportion of plants displaying guttation and the occurrence of dew were recorded. Alternative water sources were observed for a period of 4 minutes and the number of honeybees sitting and/or collecting water documented. The number of honeybees sitting on maize plants or soil surface, or foraging guttation or dew water was recorded during a 4 minute assessment period per sub-plot (2 m^2) and during the assessment in the off-crop area and in zones 0-4. Any unusual behavior (i.e. uncoordinated movements or aggressiveness) were documented and samples from colonies with disease symptoms were further investigated by the Bee Institute Kirchhain, Erlenstraße 9, 35274 Kirchhain.

During the prolonged assessment in the Baumgartenberg region, only guttation and dew occurrence and bee activity at the hives were recorded, dead bee traps were emptied and number of dead bees recorded, and the BBCH code of the maize and precipitation determined. Guttation fluid was collected only from one field. After the exposure period, the hives were relocated to a not-exposure bee yard and all colonies from both regions were assessed a last time three weeks after the end of exposure. The differences between colonies were tested with a Mann-Whitney U-test using Statgraphics Plus for Windows 3.0.

In each study region, air temperature, relative humidity and precipitation were recorded. Air temperature and relative humidity were collected every 5 minutes using a data logger at a height of 20 cm above the ground installed in each sub-region. Mistiness and precipitation were recorded daily using a rain gauge installed next to each data logger. Soil characteristics including organic carbon, particle size and soil type were determined from homogenized, sieved (2 mm) and air-dried soil cores samples (20 locations from each field), 2 cm diameter and 10 cm depth.

Analytical Methods

Guttation samples and honeybee samples were stored frozen prior to analysis. Samples for guttation fluid and honeybees from all fields were analyzed for clothianidin residues and selected samples were also analyzed for clothianidin metabolites TZNG and TZMU, and the active substance thiamethoxam (TMO), which is also used for maize seed treatment and metabolizes into clothianidin. Chromatography and detection using MS/MS (with slight modifications) were conducted according to method 00554/M001 (clothianidin, TZNG and TZMU) and method 01131 (thiamethoxam).

Bees were analyzed by weighing 1 g of bees into *ca*. 30 mL acetonitrile:water (8:2, v:v) + 1 mL/L acetic acid in a 150 mL beaker. The extraction solution was homogenized for 1 minute, the filtered solids decanted and washed with 30 mL extraction solution into 100

mL volumetric flask and brought to volume (solution not defined). An aliquot (40 mL was concentrated to the aqueous remainder and cleaned on a ChemElut (1020) column and eluted with *ca*. 80 mL cyclohexane:ethyl acetate, evaporated to dryness, then redissolved in 2 mL toluol:ethyl acetate (85:15, v:v) before applying the organic solution to a silica gel column. The residue was eluted with acetonitrile (5 mL), evaporated to dryness, and re-dissolved in 1 mL of internal standard solution (0.001 mg/L) before transferring into HPLC vial for analysis. Guttation water was analyzed without modification and the internal standard added directly to sample aliquot. Extracted bees and guttation water were analyzed for clothianidin and it transformation products TZNG, TZMU and for thiamethoxam (a precursor to clothianidin) by LSC and HPLC under reverse-phase conditions with detection by MS/MS with electrospray ionization by comparison to reference standards.

For bees, the limit of quantitation (LOQ) for clothianidin, TZNG, TZMU and thiamethoxam defined as the lowest validated fortification level was 1.0 ppb; the limit of detection (LOD) in bees was 0.3 ppb. In guttation fluid, the LOQ was 0.01 mg/L and the LOD was 0.001 mg/L.

In bees, method recovery for clothianidin ranged from 78 to 114% (mean 95 ± 9.0%, n = 29); TZNG ranged from 67 to 113% (mean 91 ± 12.0%, n = 30); TZMU ranged from 78 to 100% (mean 86 ± 7.8%, n = 30); thiamethoxam ranged from 61 to 113% (mean 89 ± 15.4%, n = 30). In guttation water, method recovery for clothianidin ranged from 93 to 137% (mean 102 ± 11.6%, n = 12); TZNG ranged from 86 to 130% (mean 98 ± 12.7%, n = 12); TZMU ranged from 95 to 131% (mean 105 ± 9.9%, n = 12); thiamethoxam ranged from 81 to 99% (mean 89 ± 7.1%, n = 12).

12. REPORTED RESULTS

Signed and dated No Data Confidentiality, GLP and Certificate of Authenticity statements were provided; a Quality Assurance statement was not provided. This study was not conducted in compliance with the Principles of Good Laboratory Practice (GLP). The data presented are an analysis of technical data submitted in support of registration and is not subject to GLP; however multiple studies from which the data was taken were conducted under GLP's. The study was submitted as a non-GLP trial.

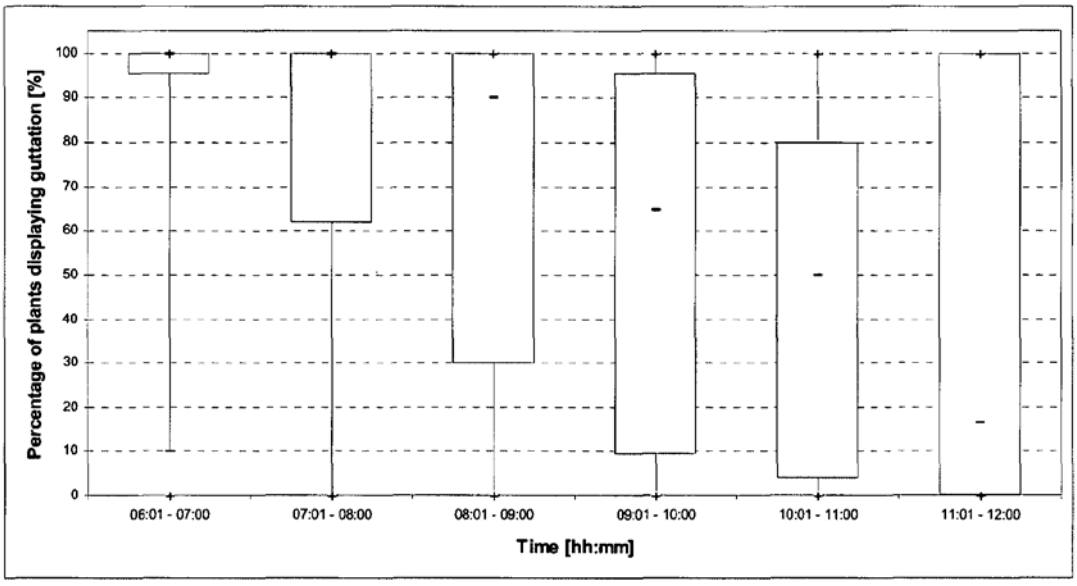
Guttation assessment

In the Baumgartenberg region, maximum temperatures during the warm phase ranged from ca. 25°C to 33°C and between 17°C and 12°C during the cold phase. There was little precipitation in April, but more regular precipitation in May with heavy period of rain on May 18th of 42 mm. In the Jennersdorf region, the maximum temperatures ranged

from 33°C to 38°C with a period of heavy rain on May 18th of 31 mm. Soil in the test fields was mainly silty or sandy loam and silty clay with a few other variations.

For both regions, guttation was observed in 97.4% of the observations days in the morning and 47.2% in the evening. The portion of maize plants displaying guttation was significantly higher ($p<0.01$; H-test according to Kruskal-Wallis) during the one hour time periods of ca. 6-7, 7-8 and 8-9 a.m. compared to all the other time periods ($p<0.01$; U-test of Mann and Whitney).

Proportion of maize plants displaying guttation during the morning.



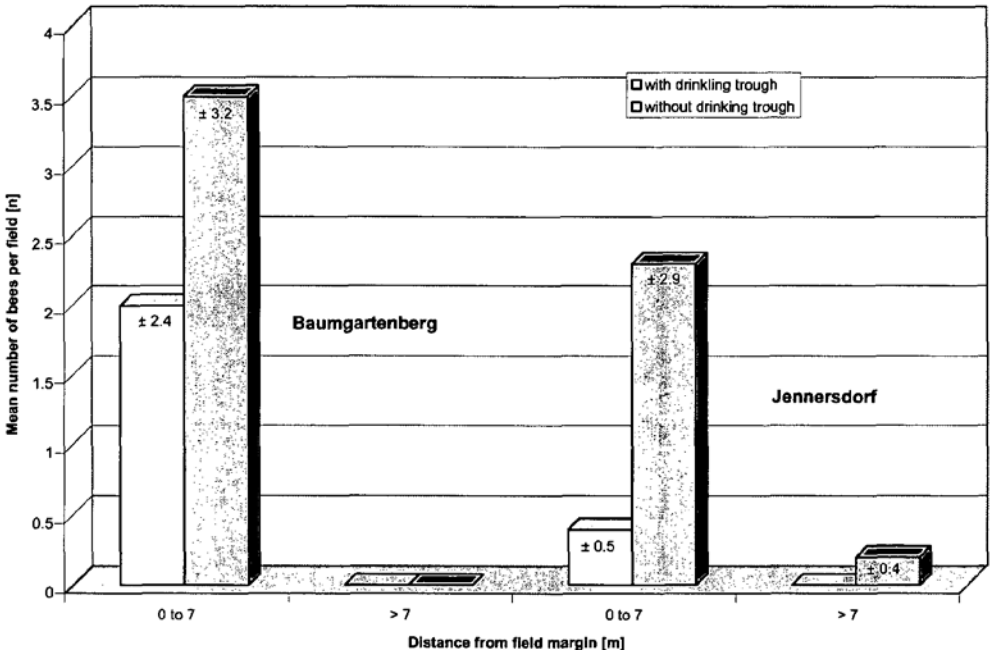
Guttation is negatively correlated with air temperature but the correlation coefficient is low ($r = -0.3$). The proportion of maize plants displaying guttation is positively correlated with the relative air humidity, but the correlation coefficient is also low ($r = 0.6$). These findings suggest that temperature and relative humidity are influencing parameters on the occurrence of guttation, but the strong degree of scattering of the values between 0-100% guttation indicates that other factors influence the occurrence of guttation.

The temperature during no guttation was a mean of 20°C, while during guttation the mean temperature was 17°C, which was significantly lower than during the absence of guttation ($p<0.01$; U-test of Mann and Whitney). Also, the mean relative humidity during no guttation was *ca*. 58%, while during guttation the mean relative humidity was 70%, which was significantly higher than during the absence of guttation ($p<0.01$; U-test of Mann and Whitney).

Guttation coincided with honeybee activity in the morning averaging 85%. The overlap between guttation and honeybee activity was lower in the evening, averaging 37%. Therefore, the coincidence of guttation of maize seedlings and honeybee activity in the morning was common and lasted up to 6 weeks after seedling emergence, while the coincidence was less regular in the evening. The overlap in the morning ranged from 10 minutes to 5.5 hours and in the evening between 10 minutes and 2.25 hours.

Honeybee visitation of the maize fields during the monitoring period was infrequent, however, on one occasion *ca.* 80% of the maize plants in zones 0-1 were visited with up to 3 honeybees per plant and coincided with conditions of intensive sunshine and an empty drinking trough. In this instance three single bees displayed signs of intoxication. If corrected for the different number of assessments per day per group, the total number of honeybees resting on the soil and maize plants was higher in the fields without a drinking trough compared to those were drinking trough was provided. Therefore it was concluded that the presence of drinking troughs reduced the number of honeybees visiting maize fields. Honeybees observed resting on maize plants were encountered almost exclusively within a distance of 7 m from the field margin suggesting honeybees preferably collect water nearest the hive vicinity. Overall, drinking troughs were observed being used by honeybees 74% of all assessments in Baumgartenberg and 55% of the time in Jennersdorf with between 0 and 100 honeybees present at the troughs at a respective check indicating again that honeybees look for water sources in the nearest vicinity of the hives.

Spatial distribution of honeybees resting on maize plants



Honeybee health monitoring

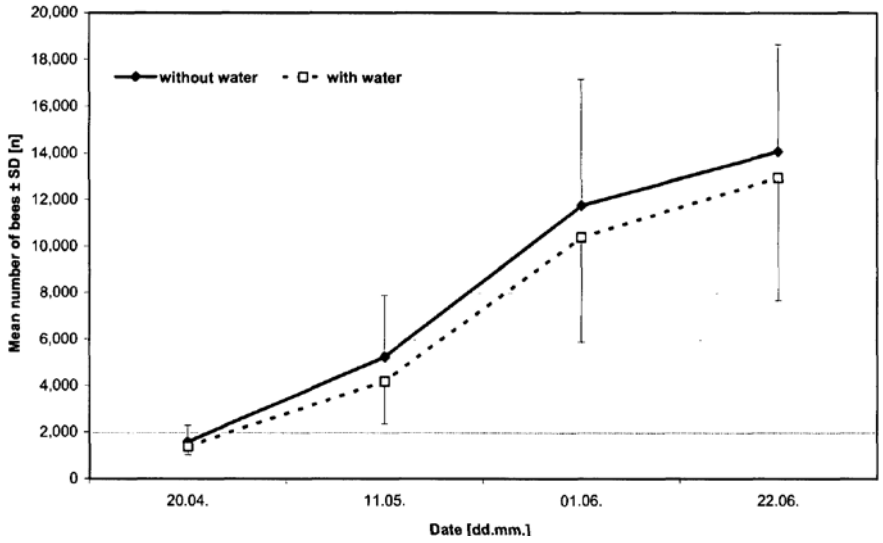
At delivery, the average colony strength was 1,370 and 2,030 bees in Baumgartenberg and Jennersdorf, respectively, rather than the 3,000-5,000 bees per colony expected. Most of the colonies were heavily infested with wax moths (*Achroea grisella*). Due to these low starting populations, and to accomplish the objectives of the study, all colonies with population strengths of less than 1,000 bees were replaced. Since inoperative colony strength was discovered during the first assessment, before emergence, the uptake of guttation fluid was not a causative factor for the low colony strength. Four out of eleven replacement colonies were also not appropriate and had to be discarded. In Jennersdorf three colonies lost the queen during transport to the site and were replaced and one colony built transversal combs and was also replaced. Due to poor starting conditions, no colony reached an average strength of a commercial bee colony at the end of the study. Only one colony reared a single queen cell and in most cases the drone brood production was low. There are further indicators of low colony strength and suboptimal foraging conditions, especially in the Jennersdorf region. Additionally, assessments in the first two fields in Baumgartenberg were terminated due to low strength of the bee colonies after colony set-up.

Colony development did not differ significantly between colonies with or without additional water source. However, significant differences were noted between the two test regions. In Jennersdorf, the effects of starvation on colonies due to unfavorable apicultural landscape conditions were clear. Even after a 3 week recover period honeybee colonies from Jennersdorf did not reach the level of those from Baumgartenberg at the end of exposure, emphasizing the importance of appropriate nutritional supply.

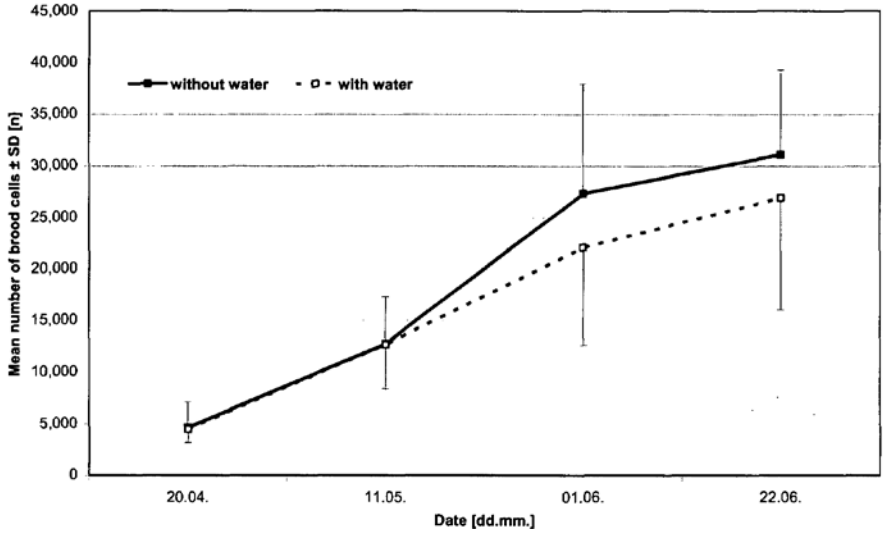
In Baumgartenberg, the initial and final colony strengths were very heterogeneous. At the end of exposure, the strongest and weakest colonies without water source were *ca*. 20,000 and 5,000 honeybees, respectively, and in the colonies with additional water source the strongest and weakest were *ca.*17,000 and 2,500 honeybees, respectively. The brood strength was in line with the population strength with some exceptions. One colony had abnormally high brood in relation to the number of adult bees, but normalized toward the end of the exposure period. Though no signs of disease were recorded, single bees were observed with intoxication symptoms. The second colony in this field showed a high infection level with *Nosema ceranae*, which can seriously affect colony development; another colony was also infected. Another colony in a separate field had patchy brood due to wax moths, but the brood pattern normalized when the colony got stronger. Finally, the number of bees in a separate colony did not increase though the brood cell development followed a normal curve. In this colony some crippled bees were observed due to medium infection with Deformed Wing Virus (DWV). Symptoms of DWV were also observed in 4 other colonies without changes in colony development. Two colonies showed clear clinical symptoms of Chronic Bee Paralysis Virus (CBPV) at the last assessment date; no further laboratory analysis was initiated.

During the 3-week post-exposure period, colony growth rate slowed in line with the natural annual growth cycle. All colonies had drone brood during the last assessment day without swarming tendencies, likely due to low colony strength compared to commercial colonies. One colony was an exception, with signs of DWV infestation and remained at low population strength. There was no difference in colony growth associated with access to drinking troughs during the exposure period ($p>0.05$; Mann-Whitney U-test).

Development of colony strength in Baumgartenberg.



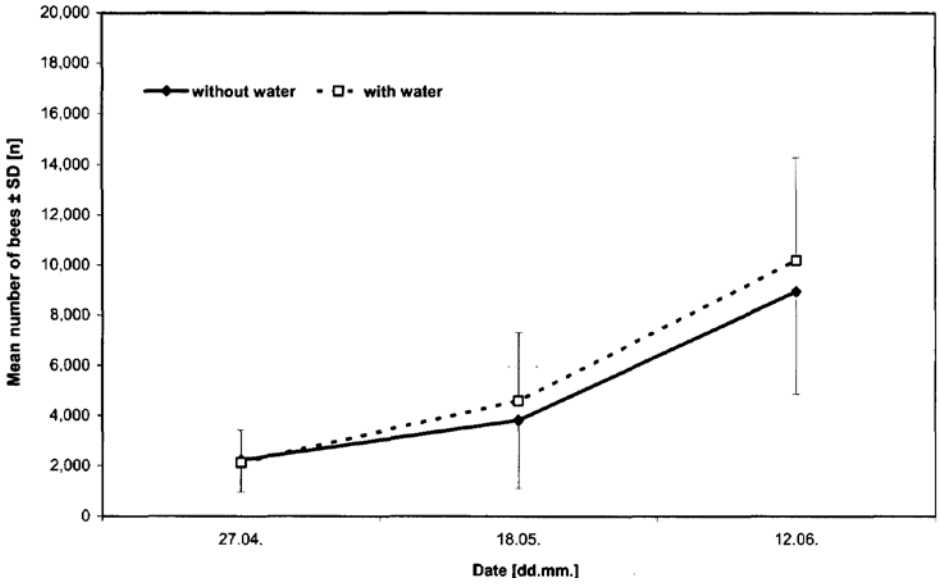
Development of worker brood in Baumgartenberg.



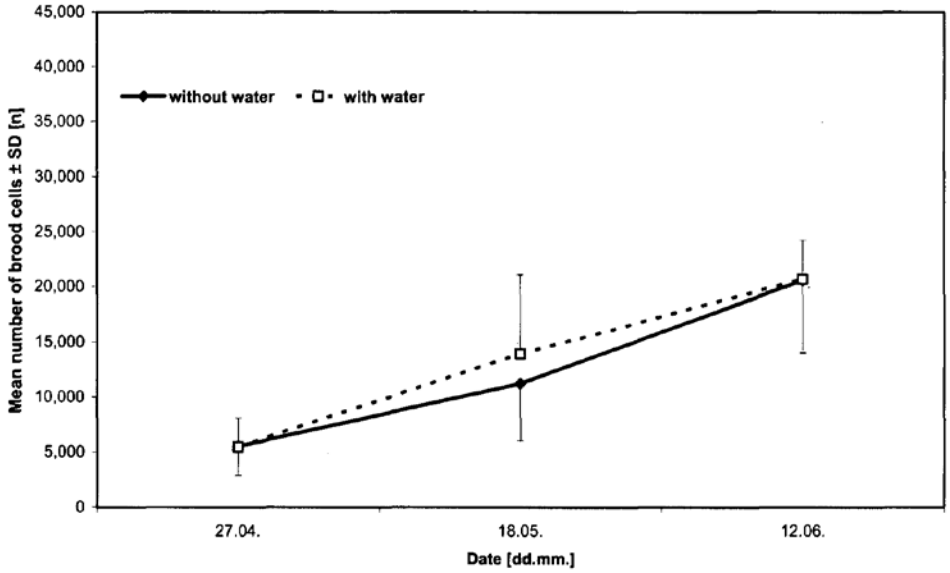
In Jennersdorf, colony development was strongly influenced by very poor foraging, nearly no natural nectar sources, therefore all colonies started to suffer starvation and had to received additional food (1.25 kg of Apifonda). However, three colonies did not start with the strength to survive the starvation period and were eliminated. The other colonies recovered and continued normal development. One colony did not grow in strength during the exposure period, but colony and brood strength remained at the initial levels and at study termination showed severe CBPV symptoms. One colony showed retarded development without any visible symptoms of disease but with an initial colony strength of only 1,500 honeybees and is regarded as inoperative for a scarce food landscape.

During the 3-week post-exposure period, colonies recovered from the starvation effects though some colonies showed severe symptoms of CBPV on the last assessment. One colony, despite a CBPV diseased queen, brood activity increased strongly during the post-exposure period. CBPV symptoms were seen in two other colonies, though only worker bees were affected and in one of the colonies brood activity decreased rapidly though the proportion of adult workers and brood remained normal. Another colony did not recover in the same way as the others following relocation to the non-exposure bee yard, it grew and tripled its number of bees in the three week post-exposure period, confirming the assumption that starvation was a key factor impeding development during the exposure period. The significantly better apicultural conditions in the post-exposure region were reflected in the presence of drone brood in most colonies on the last assessment. Swarming tendency was again not observed likely due to low colony strength. Also, as with the other region, there was no difference in colony growth associated with access to drinking troughs during the exposure period ($p>0.05$; Mann-Whitney U-test).

Development of colony strength in Jennersdorf.



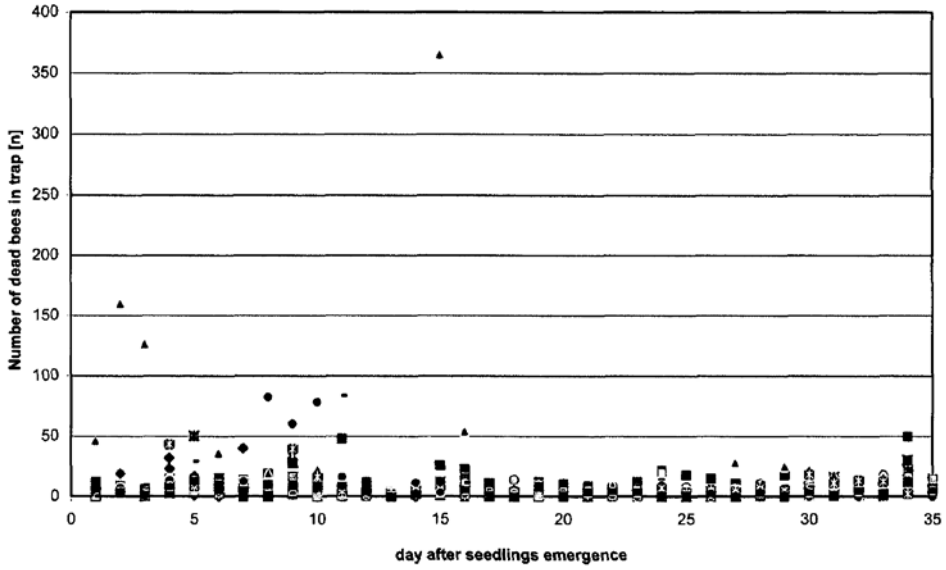
Development of worker brood in Jennersdorf.



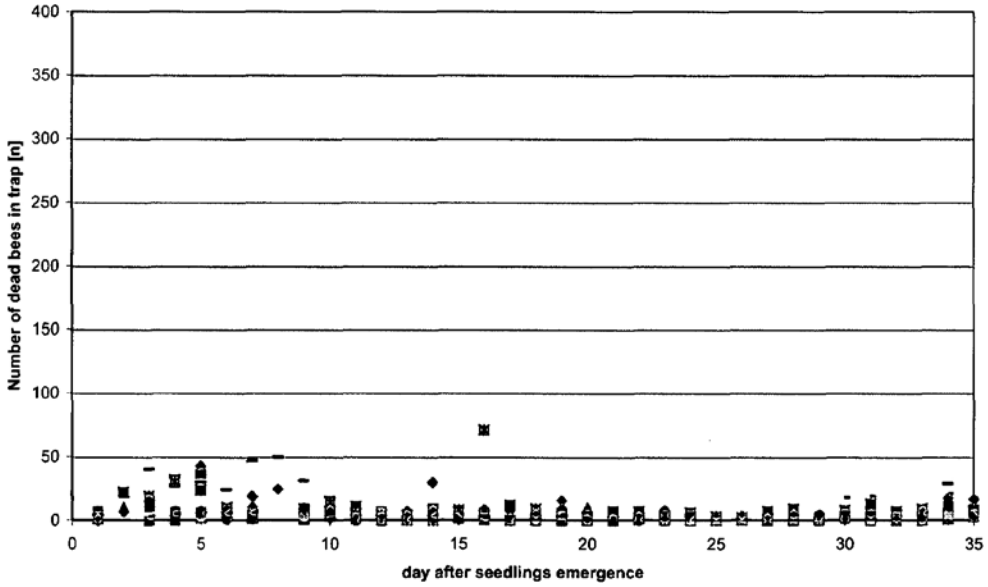
The number of dead bees in the trap fluctuated during the study period and between colonies. In both regions, the mortality was ≤5 bees per hive during 70% of the assessment days and ≤20 bees per hive during 95-96% of the assessment days. However, there were periods of mortality peaks in single colonies.

In Bumgartenberg, higher mortalities were noted in the first half of May and the first days of June. In one colony a total of 332 bees were in the trap in the first three days of May and 365 dead bees were found on May 15 (*ca*. 30 days after drilling in mid April); however, colony development was not affected. In two other fields/colonies, a maximum of 50 and 82 dead bees were found on May 6. In Jennersdorf, peak mortalities were recorded on May 7-8 and 15-17 in single colonies. One of these colonies had a maximum of 144 dead bees on May 15; however, colony development was not affected. Three colonies had to be removed from the fields due to strong starvation stress and weak initial populations (715, 715, and 455 bees); therefore, the dead of these colonies were attributed to food scarcity and low population strength at the start of the study.

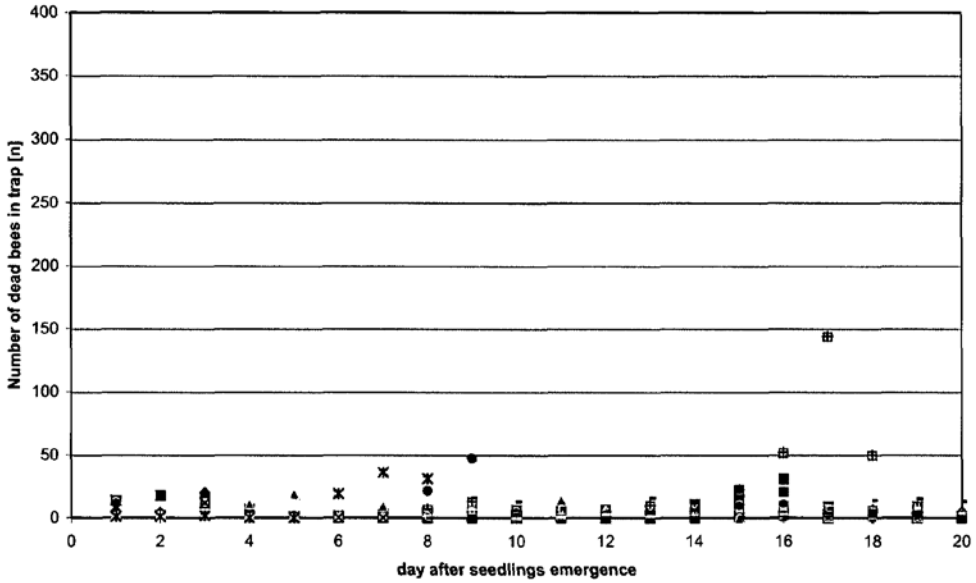
Mortality in dead bee traps in colonies **without** access to additional water source in Baumgartenberg



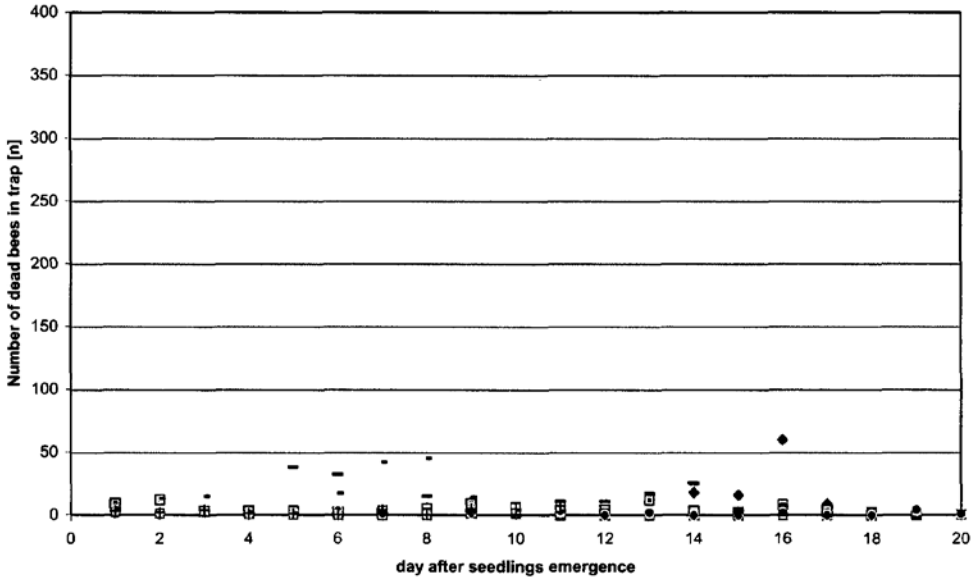
Mortality in dead bee traps in colonies **with** access to additional water source in Baumgartenberg



Mortality in dead bee traps in colonies **without** access to additional water source in Jennersdorf



Mortality in dead bee traps in colonies **with** access to additional water source in Jennersdorf



The number of days with greater mortality is more frequent for hives with no access to an additional water source ($Chi^2 = 5.8$, $p<0.05$). However, this correlation was not found for both regions. In Baumgartenberg, the difference between colonies with and without drinking troughs was highly significant ($Chi^2 = 10$, $p<0.001$), but there was no significant difference observed for Jennersdorf ($Chi^2 = 0.02$, $p>0.05$).

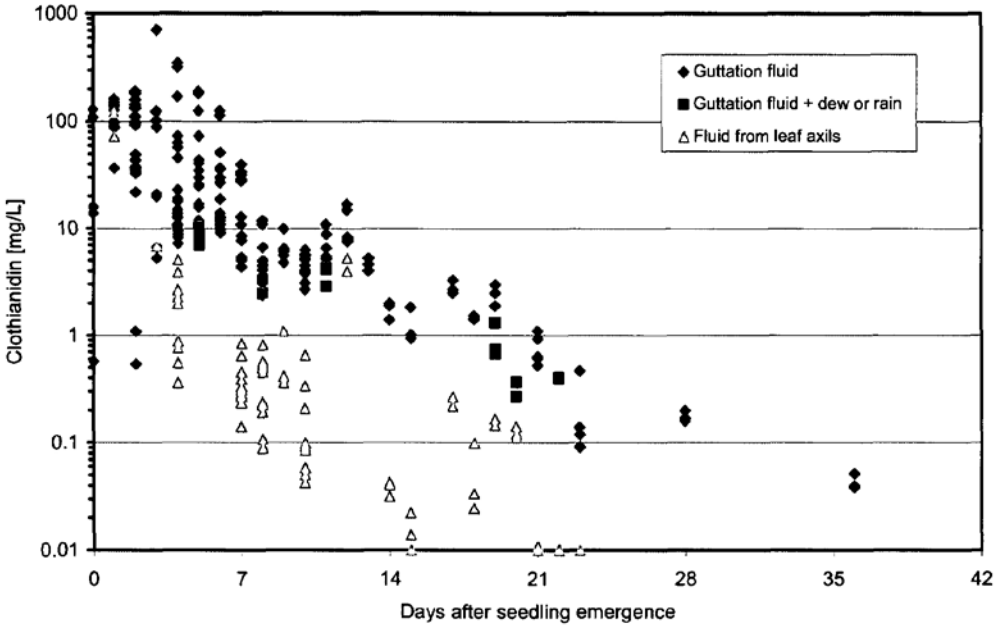
In Baumgartenberg, 172 dead bees were analyzed (126 from colonies without water trays and 46 with water trays). Additionally, 2 samples of the May 2 (>160 dead bees) and May 15 (365 bees) from one colony were divided and subsampled to check for homogeneity of residues. Residues were detected in both samples with and without additional water sources; however, residue levels were not correlated with the number of dead bees sampled in the dead bee trap. In Jennersdorf, 57 dead bees were analyzed (38 from colonies without water trays and 19 with water trays). Residues were found in colonies with and without water trays; however, residues were again not correlated with the number of dead bees in the traps.

Only a few bees were observed resting or walking on the maize seedlings or surrounding soil; however, single bees were observed with intoxication symptoms on the fields coinciding with the day of highest peak of mortality. In Baumgartenberg, both colonies from one field showed increased aggressive behavior during the exposure period, which decreased when the colonies were removed to the non-exposure bee yard. Paralyzed bees, apathic behavior, inability to fly, cramps and spasms were also observed in this field. In another field, bees were also observed with intoxication symptoms correlated with peak mortality. During the 6 week period single bees with intoxication symptoms were observed in 7 field (affected bees n = 2-16). No correlation was found between these observations and mortality rate. In Jennersdorf, only single bees with intoxication symptoms were observed with no correlation to elevated mortality. During the 3 week exposure period one bee each was observed with symptoms like apathy, tremor and uncoordinated movement in 3 fields.

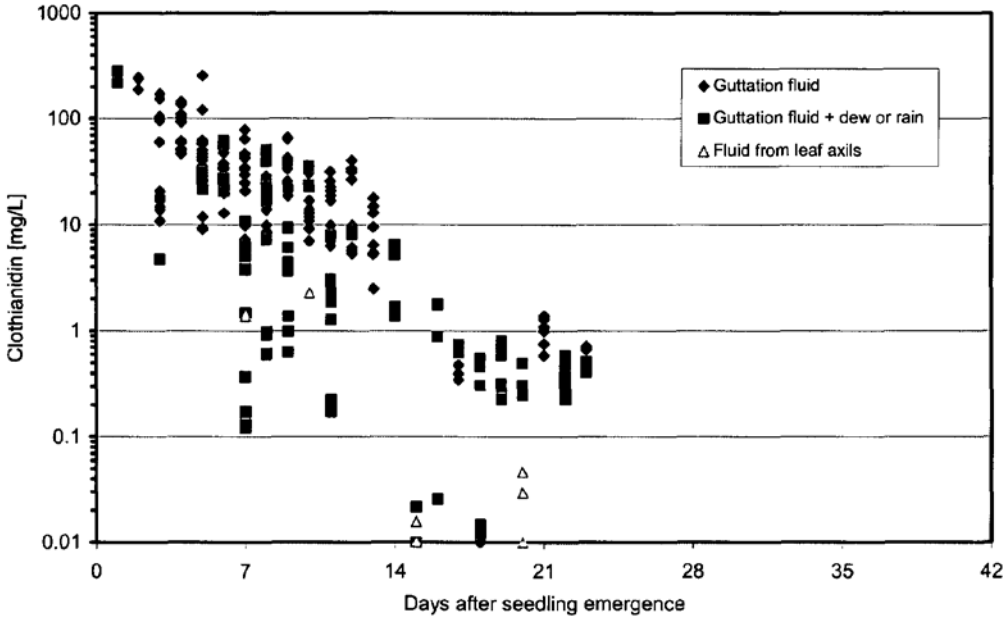
Residue Levels

Some of the guttation fluid samples were assumed to be a mix of guttation fluid, dew and/or rain. Residue analysis indicated initial concentrations for both regions were between 100 to 200 mg/L clothianidin. Samples suspected of being diluted with dew or rain resulted in similar or lower concentrations compared to samples of pure guttation fluid and concentrations in samples collected from leaf axils were even lower. Residue levels in all samples declined exponentially over time with concentrations in guttation fluid of about 1 mg/L three weeks after emergence and 0.1 mg/L within five weeks.

Clothianidin residue levels in guttation fluid from maize plants in Baumgartenberg

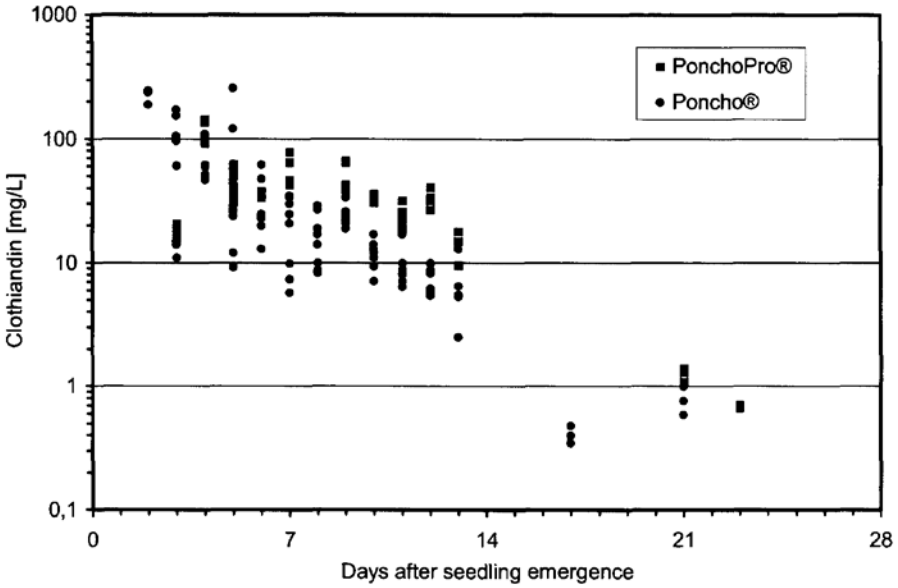


Clothianidin residue levels in guttation fluid from maize plants in Jennersdorf



Initial residue levels were similar for seedlings treated with Poncho or Poncho Pro; however there was a slight delay in residue decline for seedlings treated with Poncho Pro compared to Poncho.

Clothianidin residue levels in guttation fluid from maize plants in Jennersdorf in relation to application rate (comparing fields with application of Poncho and Poncho Pro).



In Baumgartenberg, clothianidin residues in bees were between <LOD and 45.5 ppb and in guttation water were between <LOQ and 717 mg/L. TZNG residues in bees were between <LOD and 31.2 ppb and in guttation water were between <LOD and 4.0 mg/L. TZMU residues in bees were between<LOD and 3.3 ppb and in guttation water were between <LOD and 9.0 mg/L. TMO residue levels in bees and guttation water were always below LOQ.

In Jennersdorf, clothianidin residues in bees were between <LOD and 384.9 ppb and in guttation water were between <LOQ and 285 mg/L. TZNG residues in bees were between <LOD and 39.7 ppb and in guttation water were between <LOD and 4.9 mg/L. TZMU residues in bees were between<LOD and 12.4 ppb and in guttation water were between <LOD and 6.7 mg/L. TMO residue levels in bees were always below LOQ and in guttation water were between <LOD and 0.054 mg/L.

Study Author Conclusions

Guttation in maize seedlings occurred regularly and the presence of guattation fluid usually overlapped with bee flight activity. Initial residues of clothianidin in guttation fluid from treated seeds were in the range of 100 to 200 mg/L, but decreased exponentially in the following weeks, reaching levels of 1 mg/kg after 3 weeks and were below 0.1 mg/L after 5 weeks. Bee colonies were very adaptable and developed

reasonably well despite low population strength at the start of the experiment. Even transitory starvation stress in Jennersdorf did not ultimately affect the majority of managed colonies. However, low population strength in the early phase, suboptimal forage conditions, and some infestation of diseases made these colonies more susceptible to chemical stress factors than typical commercial colonies in more appropriate apicultural conditions. Nevertheless, colony development was not affected by exposure to guttation in Poncho- or Poncho Pro-treated maize seedlings. After removing the colonies from experimental conditions, development continued as expected with colonies from Jennersdorf region starting to recover from food scarcity. Therefore, the study authors concluded that no visible harmful effect on the colony level due to exposure to guttating maize fields sown with clothianidin-treated seeds. The honeybees were able to develop properly even under unfavorable conditions of the study.

13. REVIEWER'S COMMENTS

The reviewer agrees with the study author that exposure of honeybees to guttation fluid from clothianidin-treated maize seeds had no harmful effects at the colony level over the test period of 3 or 6 weeks. However, the test material did have detrimental effects on individuals with one case of increased aggression in two colonies from one field and some evidence of colony weakening due to increased infestation and disease. Furthermore, the study period may not have been adequate to accurately determine colony effects over time under field conditions in which exposure may have occurred for a longer period of time, and recovery under ideal conditions of a non-exposure bee yard are not available.

14. REFERENCES

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